ON CERTAIN PHYSIOLOGICAL DIFFERENCES BETWEEN DIFFERENT PREPARATIONS OF SO-CALLED "CHEMICALLY PURE" SODIUM CHLORIDE

MARY MORRISON WILLIAMS AND M. H. JACOBS

(From the Marine Biological Laboratory, Woods Hole, Massachusetts, and the Department of Physiology of the University of Pennsylvania)

I

It is the purpose of the present paper to direct the attention of biologists to important differences in the toxicity to living cells and organisms of certain commercial brands of so-called C.P. sodium chloride which have usually been treated in the past as being more or less identical chemically. The brands in question have all been used frequently at the Marine Biological Laboratory and other scientific institutions in this country; and, in view of the striking differences that will be shown to exist between them, the question arises how far the work of different investigators, who have in the past used sodium chloride of unspecified origin, is comparable and, indeed, how far many published statements concerning the physiological properties of this salt in pure solutions may be generally true. While these questions cannot as yet be answered with entire certainty, the necessity is clearly indicated for much greater care in the future than has been exercised in the past in physiological work involving this commonest of all salts.

The observations which formed the beginning of this investigation were made more or less accidentally in connection with certain unpublished studies on the hemolytic effects of ammonium chloride on the erythrocytes of the various classes of vertebrates, particularly the fishes. In the course of these studies, controls of isotonic NaCl were used for comparison, the salt employed being that which happened at the time to be in general use at the Marine Biological Laboratory. It soon became apparent that whereas the erythrocytes of the mammals remained intact almost indefinitely in such control solutions, those of several species of fishes, among them the sea robin, the butterfish, the cunner, the tautog, the mackerel, the scup and the fresh water perch, underwent destruction in times ranging from a few minutes to several hours, though failing to do so in similar solutions of KCl or $CaCl_2$ or in properly diluted sea water.

The unique behavior of NaCl is brought out in Fig. 1, in which are

plotted against the times in hours from the beginning of the experiment the cell counts, obtained by the usual hemocytometer method, of suspensions of the erythrocytes of the sea robin (*Prionotus carolinus*) in approximately isotonic solutions of KCl, NaCl and $CaCl_2$ and in a physiologically balanced mixture of the three salts. The rapid destruction of the erythrocytes here shown in solutions containing NaCl and their preservation in the other solutions are entirely typical of dozens of experiments made during the summer of 1926 with the par-

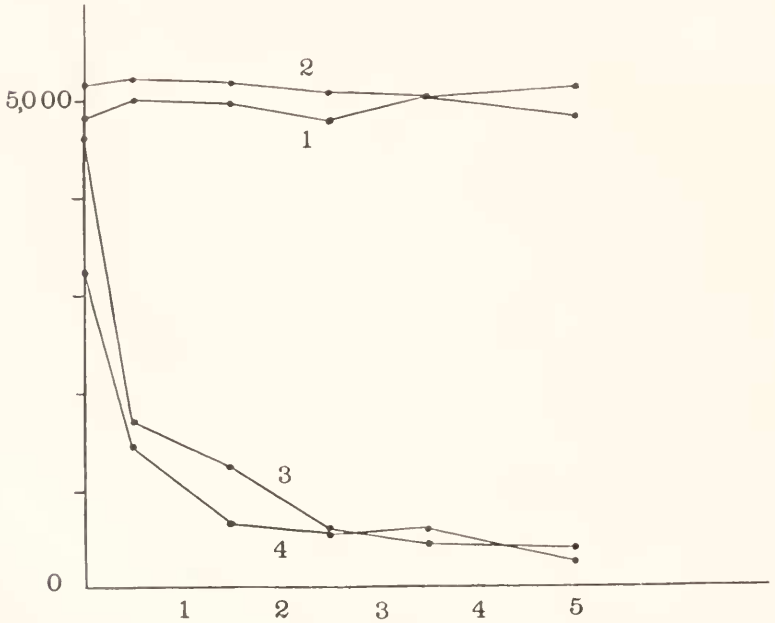

FIG. 1. Effect of exposing erythrocytes of the sea robin (*Prionotus carolinus*) to: (1) M/3.7 KCl, (2) M/5.5 $CaCl_2$, (3) M/3.7 NaCl and (4) a mixture of these solutions in the proportions of 2 : 2 : 96. Ordinates represent numbers of cells per cubic millimeter and abscissæ times in hours.

ticular brand of salt in question, not only on the erythrocytes of the sea robin but on those of the other species mentioned above as well.

On repeating the experiments the following year our surprise was great when the expected hemolysis in NaCl solutions completely failed to appear, the erythrocytes remaining intact in such solutions for many hours with no more evidence of injury than when KCl or properly diluted sea water was employed. The only difference between the two sets of experiments was that by chance a new brand of C. P. NaCl had been substituted in 1927 for that used in 1926. On going back to the former brand the earlier results could again be repeated at will. Evi-

dently there was in respect to their hemolytic properties at least, a very decided difference between two preparations of NaCl, both presumably of good quality and both in common use at the Marine Biological Laboratory and elsewhere. Because of the possible importance of such differences in physiological work, further experiments on fish erythrocytes were therefore undertaken with the more common commercial brands of C.P. sodium chloride; and the results were later extended to several other types of living material. The general outcome of these experiments may now be described.

II

In all, five brands of C.P. NaCl, each prepared by a different manufacturer, were studied. In every case, samples from several separate and previously unopened containers were used. In order to avoid any possibly unjust conclusions being drawn as to the relative values of the salts of the different manufacturers for the chemical purposes for which they were primarily intended, the different brands will be designated merely by the letters *A* to *E*, inclusive. It is perhaps not improper to say that the brand designated by *A*, which is the least harmful to fish erythrocytes of all those studied, being in fact practically as harmless as KCl, is the Kahlbaum salt of the best quality obtainable. Of the other four brands, *B* was at times almost as good as the Kahlbaum preparation, but at other times was distinctly harmful, the differences observed depending partly on the lot of salt used and especially on the species of fish furnishing the erythrocytes. In our earlier experiments, in which the decidedly resistant erythrocytes of the sea robin were employed, this brand was almost indistinguishable from *A*, but in later observations made by Dr. A. K. Parpart, working with one of the authors on another problem, it appeared that it was quite incapable of preserving for any length of time the much less resistant erythrocytes of the tautog and the cunner which were, however, not markedly injured by brand *A*. Brands *D* and *E* were invariably destructive to all the fish erythrocytes studied, though more rapidly so to some than to others. Brand *C*, as far as it was studied, appeared to be relatively harmless, but our information about it is not very complete.

A typical experiment in which the effects on the erythrocytes of the scup (*Stenotomus chrysops*) of brands *B*, *C*, *D* and *E* and of KCl is illustrated in Fig. 2. The blood in this case, as in all others here reported, was freshly obtained from a living fish without the use of any anticoagulant and was added immediately to the solutions in question in the proportion of approximately 1 to 200 by volume (*i.e.*, one drop to 10 cc.). A slight variation in the sizes of the drops of blood was of no

significance, since cell counts were made in every case. In the absence of exact information concerning the osmotic pressures of the various bloods studied, the concentration of NaCl employed was taken, unless otherwise indicated, as 0.25 M. Such solutions have a freezing point of approximately −0.86° C., which is not very far removed from that of the plasma of the various marine teleost fishes for which figures are available; and at all events the concentration was the same for the various brands of salt employed, so that the results were entirely comparable among themselves.

It will be noted in Fig. 2 that for the duration of the experiment

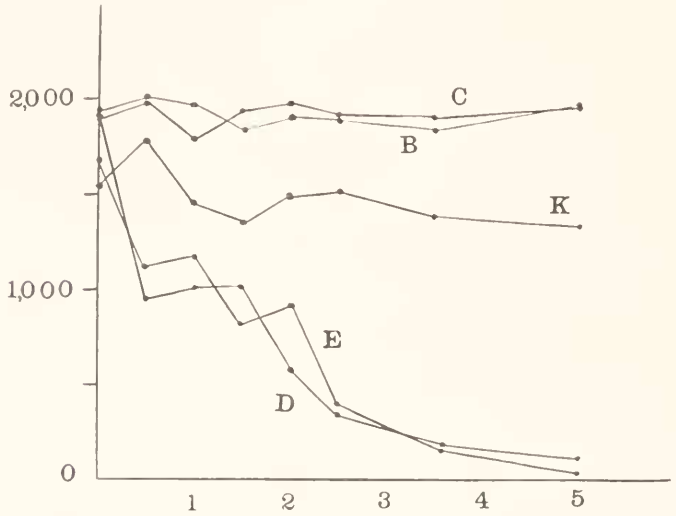

FIG. 2. Effect of exposing erythrocytes of the scup (*Stenotomus chrysops*) to M/4 solutions of brands *B*, *C*, *D* and *E* of NaCl and to M/4 KCl. Ordinates represent numbers of cells per cubic millimeter and abscissæ times in hours.

(5 hours) there was no appreciable decrease in the number of erythrocytes in solutions of brands *B* and *C*, while in similar solutions of brands *D* and *E* the numbers had decreased very appreciably within one half hour and very few erythrocytes remained at the end of four hours. It may be mentioned incidentally that the erythrocytes of the scup, like those of the sea robin, are relatively resistant ones; those of the butterfish or of the cunner disappear far more rapidly.

This particular experiment is typical of several dozen others differing in detail but all giving essentially similar results. In addition, many incidental observations by W. A. Smith, S. E. Hill and A. K. Parpart working with one of the authors on other problems in which cell counts were not made but hemolysis was followed by a macroscopic

method have been in entire agreement with the results pictured in Fig. 2. It may, therefore, be considered as definitely established that the erythrocytes of certain fishes are affected in an entirely different manner by various preparations of C.P. NaCl in common use.

III

As to the cause of these differences, two main possibilities suggest themselves: either pure sodium chloride is in itself destructive to the erythrocytes and its harmful effect is antagonized by impurities of some sort present in brands *A* and usually in brands *B* and *C*, or pure sodium chloride is in itself relatively harmless to this form of material and the injury is due to a toxic impurity of some sort in brands *D* and *E* and sometimes in *B* and *C*. Though the first type of explanation might perhaps appear to be somewhat far-fetched, it must not be forgotten that pure NaCl has been generally considered to be highly toxic (Loeb,

TABLE I

Effect on erythrocytes of the freshwater perch of solutions of NaCl of brands B and E before and after recrystallization. The figures represent numbers of erythrocytes in 1 cubic millimeter of a dilute suspension.

Number of Experiment	Brand B				Brand D			
	Original Salt		Recrystallized Salt		Original Salt		Recrystallized Salt	
	Beginning of Experiment	After 1 hour	Beginning of Experiment	After 1 hour	Beginning of Experiment	After 1 hour	Beginning of Experiment	After 1 hour
1	245	200	200	190	225	0	240	0
2	230	225	225	205	175	0	150	0
3	200	250	250	160	150	0	150	0
Average	225	225	225	218	183	0	180	0

1900; Osterhout, 1922) and that its toxic effects may be antagonized by very low concentrations of plurivalent cations—for example, in the case of the cilia of *Mytilus*, according to Lillie (1906), M/51,200 $FeCl_3$ is strikingly antitoxic.

It was thought that some light might be thrown upon these two alternative types of explanation by a comparison of the effects upon the same material of a harmful and a harmless brand of salt before and after recrystallization. Brand *B* was known, for example, to be harmless and brand *E* to be highly destructive to the erythrocytes of the freshwater perch. If the first of the two types of explanation were correct, recrystallization should tend to make brand *B* more toxic than before and leave *E* unchanged; if, on the other hand, the second were correct, recrystallization should make *E* less toxic and leave *B* unchanged.

Lack of time prevented extensive recrystallizations from being carried out, but in Table I are represented the results of one experiment in triplicate of this sort. Because of the difference in the osmotic pressure of the blood of freshwater as compared with marine teleosts, the concentration of NaCl here employed was 0.147 M, which has a freezing point in the vicinity of that found by Garrey (1916) for the blood of a number of freshwater fishes, *i.e.*, approximately −0.50° C. It will be noted that the experiment shows no significant change in the properties of either salt after recrystallization. It is therefore inconclusive, so far as throwing light upon the nature of the differences in the physiological properties of the salt preparations in question is concerned, but it does indicate one fact of great practical importance, namely, that any impurities that may be present are difficult to remove by recrystallization.

The question of possible antagonism was more directly and extensively attacked in another way. Since it is well known from the work of Loeb and others that perhaps the most effective single antagonist of the toxic effects of sodium is calcium, and that solutions containing sodium, calcium and potassium in the proper proportions form for most cells and tissues a fairly good substitute for their natural medium, attempts were made to find some combination of the chlorides of calcium or of calcium and potassium, with the toxic brands of sodium chloride that would remove or at least greatly diminish the hemolytic effect of the latter. In this we were completely unsuccessful. In particular, the addition to the toxic brands of NaCl of $CaCl_2$ and KCl in the approximate proportions in which they occur in the body fluids of the vertebrates or in sea water was almost without effect (see Fig. 1). Only when isotonic solutions of $CaCl_2$ or KCl or both were added to similar solutions of NaCl in sufficient quantities to dilute the latter appreciably did a diminution of the hemolytic effect become apparent. This effect, however, which is entirely different from antagonism, is what would be expected if the NaCl carried a toxic impurity.

It has been mentioned above that the erythrocytes of marine fishes are preserved fairly normally in properly diluted sea water, which is a well-known example of a physiologically balanced salt mixture. In several experiments, diluted sea water was mixed in different proportions with approximately isotonic solutions of one of the toxic brands of NaCl. In such experiments it was found that the hemolytic effect of the added salt could, in general, be detected to an extent that depended upon its concentration in the mixture. This result is again what would be expected if a toxic impurity were associated with the sodium chloride.

In view of the fact that all attempts to demonstrate a physiological antagonism between the toxic brands of NaCl and various calcium and potassium mixtures failed completely, the view was definitely abandoned that hemolysis by some salt preparations is due to the destructive effects of pure NaCl itself. The fact that brand *B* could manifest its harmful effects even in the presence of a considerable excess of diluted sea water and the additional fact that brand *A*, which has been consistently harmless, is at the same time one generally considered by chemists to be of especially high purity seem to point rather to something added to the sodium chloride in the toxic samples of the salt. It may be mentioned that Dr. Eric G. Ball has recently obtained evidence of a very direct and convincing nature that the hemolytic effects of some brands of NaCl are due to contained impurities. This evidence will soon be published elsewhere.

Accepting the view that some brands of C.P. NaCl contain an impurity highly destructive to the erythrocytes of fishes there may be mentioned briefly several of our unsuccessful attempts to determine the nature of this impurity. Partly because our results on this point were completely negative and partly because of the much more extensive observations along the same lines soon to be published by Dr. Ball, it will be sufficient here merely to eliminate from further consideration one or two conceivable factors.

It is known that the erythrocyte is, in general, fairly sensitive to pH changes and also that some preparations of so-called "neutral salts" are not entirely neutral. One of the first of the possibilities to be considered, therefore, was the reaction of the various solutions studied. It was found that as far as pH measurements can be made upon completely unbuffered solutions there were no significant differences in reaction between the different sodium chloride solutions and the distilled water used to make them up, or between these solutions and similar ones of completely harmless KCl. Furthermore, in one experiment there was added to solutions of brands *B* and *E* sodium bicarbonate in the proportion of one part of M/4 bicarbonate to twenty of M/4 NaCl. The pH of the resulting mixtures was then adjusted to 7.0 in each case by the addition of carbon dioxide in the proper amounts, a procedure which leaves the effective osmotic pressure of the mixture for the erythrocyte unaltered. Blood was then added to these well buffered mixtures, which were kept tightly stoppered throughout the remainder of the experiment. In spite of this careful regulation of the pH of the solutions, the erythrocytes underwent destruction in the presence of NaCl of brand *E* and remained intact in the case of brand *B* exactly as before. In still other experiments, it was shown that with a given

salt a change in the reaction of the solutions of two pH units (*i.e.*, from pH 6.0 to 8.0), which greatly exceeds any differences that could conceivably have been present in any of our experiments, had negligible effects upon the characteristic properties of the salts. It may be considered fairly certain, therefore, that the physiological differences between the salts in question are not due to pH effects.

In our search for possible impurities in sodium chloride preparations it was suggested to us by a chemical colleague that fluorides, which are fairly toxic to some living cells, might perhaps be concerned. Experiments were therefore made in which sodium fluoride was added in different proportions to the harmless salt of brand *B*. The proportions used ranged from a maximum concentration of NaF of 0.025 M by a series of dilutions with a factor of one fifth to a minimum concentration of the order of 0.00000001M. In none of these solutions, however, was brand *B* caused to resemble even remotely brands *D* and *E*, and it was therefore concluded that fluorides could scarcely be the impurity concerned. Similar experiments were carried out with salts of several toxic metals such as Pb, Hg and Cu which might conceivably have been present in traces in the more injurious salt preparations, but our results were again essentially negative.

As far as it was possible to carry our experiments up to the time when it became necessary to discontinue them in 1928, absolutely no clue had been obtained as to the nature of the hypothetical impurity. It should be emphasized, however, that a lack of knowledge of the nature of this impurity in no wise detracts from its physiological importance or renders its disturbing effects in certain types of experimental work less real.

IV

After establishing the fact that certain brands of so-called C.P. NaCl are highly destructive to the erythrocytes of a number of teleost fishes, experiments were undertaken to determine how far similar effects could be obtained with other forms of living material. It is evident that effects of this sort might, if unrecognized, cause considerable confusion in physiological work, particularly since all the brands of sodium chloride in question have been commonly used in such work—frequently with no published statements by which they may be identified. Additional experiments were therefore undertaken upon the following forms of material: mammalian erythrocytes, newly-hatched *Fundulus*, the eggs of *Arbacia* and the cilia of *Mytilus*. These experiments may be briefly described in the order mentioned.

As contrasted with the erythrocytes of the fishes, those of the mammals appear to be little injured by any of the brands of sodium chloride

in question. It is doubtless owing to the comparative insensitiveness of this much-studied type of cell that the striking physiological differences in the properties of different salt preparations did not long ago become generally known. Our experiments were carried out on the blood of man, the ox, the dog, the cat, and the porpoise in the manner described above, the only difference in technique being that the concentration of the salt employed with the mammalian erythrocytes was 0.154M instead of 0.25M.

The results in the case of every mammal studied were, briefly, that for 10 or more hours at room temperature or for 24 hours partly at room temperature and partly in a refrigerator there was no appreciable hemolysis in any of the solutions. In experiments of longer duration, there were in a few cases some slight indications of differences in the expected direction, but these were so small and irregular as to be of little significance. It is possible that by employing aseptic precautions, which were not practicable in our experiments, and by keeping the erythrocyte suspensions for several days, constant differences might be demonstrated. For practical purposes, however, in ordinary experiments of short duration with mammalian blood it would appear to make little difference which brand of sodium chloride is used.

The experiments made upon *Fundulus heteroclitus* are of interest because it was upon this material that Loeb (1900 and later papers) obtained his most striking evidence of the toxicity of pure sodium chloride. Though for a number of reasons it appeared to be impossible that the effects described by Loeb could have been due to an impurity in the salt used rather than to the salt itself, it nevertheless seemed of some importance to determine whether with *Fundulus* the primary toxicity of pure sodium chloride might be modified in any way by the contaminating impurity supposed to be present in some preparations. The general result of our experiments was to show that this is, in fact, the case.

A typical experiment on newly-hatched, free-swimming fish is described in Fig. 3. In it, brands *B* and *E* were compared with respect to their ability to stop (*a*) the swimming movements of the animals and (*b*) the heart-beat. The concentration was in each case M/2, which is approximately isosmotic with Woods Hole sea water. It will be observed that the differences between the two salts are rather striking. At the end of 6 hours nearly all swimming movements had ceased in the animals exposed to brand *E*, while only a few of the individuals exposed to brand *B* had been similarly affected; some continued to move in this solution for over 12 hours. The cessation of the heart-beat also occurred much more rapidly in the presence of brand *E* than

in that of brand *B*.* These differences were observed many times with no exceptions. It may be concluded, therefore, that the observed effects of sodium chloride upon *Fundulus* depend to a considerable extent on the particular salt preparation employed.

A very sensitive test object for many purposes is the egg of *Arbacia*, whose rate of cleavage is affected in a readily measurable manner by very slight changes in, for example, the osmotic pressure and the carbon dioxide tension of the surrounding medium. Since pure isotonic sodium chloride is known to be toxic to this egg, it was thought that differences in the properties of different salt preparations might be

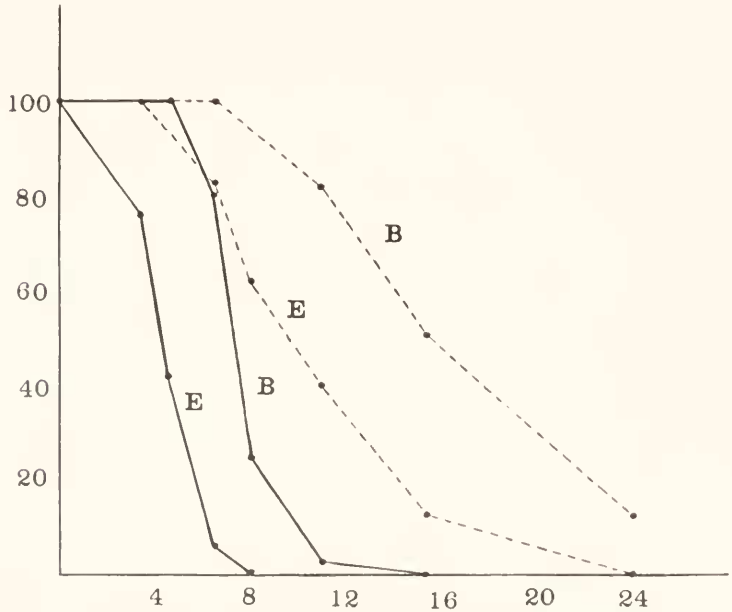

FIG. 3. Effect on newly-hatched *Fundulus* of M/2 NaCl of Brands *B* and *E*. Ordinates represent percentages of the animals showing swimming movements (solid lines) and heart-beat (broken lines); abscissæ represent times in hours.

shown by exposing fertilized eggs of *Arbacia* to them for suitable times and then determining the effect of such exposures upon the rate or the final percentage of cleavage. This was done in two ways: first by placing the eggs in the sodium chloride solution to be tested shortly before cleavage and allowing them to remain in the solution, and second by employing a short temporary exposure to the salt followed by a return to sea water. The first type of experiment proved to be entirely unsuitable owing to the failure of the eggs to divide at all, but the second type yielded results which, while not wholly satisfactory, were at least suggestive.

The general result obtained from experiments of this latter type was that in some cases there were no very significant differences between the effects of salts *B* and *E*, but that in several cases where decided differences appeared, these were always in such a direction as to indicate a greater toxicity of brand *E* than of brand *B*. The reverse condition was never obtained. An experiment showing a very considerable difference in the toxicity of salts of brands *B* and *E* is summarized in Table II. It may scarcely be considered a typical experiment, however, since the differences observed were usually not so great.

Finally, a few observations were made upon the cilia of *Mytilus*, which Lillie (1906) has shown to be very rapidly injured in solutions of pure isotonic sodium chloride. It is, of course, difficult to treat the beat of cilia in a strictly quantitative manner, since different groups

TABLE II

Effect on Subsequent Cleavage of Exposure of Fertilized Arbacia Eggs to Two Brands of Sodium Chloride

Length of Exposure	Percentage Undergoing Cleavage within 2 Hours	
	Brand B	Brand E
minutes		
5	68	3
10	39	4
15	4	1
20	2	5
25	8	2

come to rest at different times and even within the same group certain individual cilia continue to beat long after the others have ceased to do so. It is possible, therefore, for the experimenter merely to estimate in a general way when some given end-point has been reached. As far as such estimates could be made, our experiments showed no significant differences between the different brands of sodium chloride, perhaps because in this case the pure salt itself is extremely toxic. For these particular experiments brand *A* was not available, but a sample of *B* of very low toxicity to fish erythrocytes was compared with brand *E* of high toxicity. The times for the attainment of the same estimated end-point with different gill filaments were found to be 15, 13, 4.5, 11 and 9.5 minutes (average 10.6 minutes) with brand *B;* and 11, 13, 7 and 10.5 minutes (average 10.4 minutes) with brand *E*, respectively. It is not impossible that more extensive and refined experiments would be capable of demonstrating definite differences, but as far as the present evidence goes, these would not likely be very great.

Summarizing the results of the various experiments with different types of living material, it may be said that no significant differences between the different brands of sodium chloride studied have been found with mammalian erythrocytes or with the cilia of *Mytilus;* occasional but by no means constant differences in the expected direction have been found with the eggs of *Arbacia*, constant differences of considerable magnitude in the same direction occur with newly-hatched *Fundulus;* and differences of the most striking and characteristic sort are invariably present in the case of the material which was first studied, namely, the erythrocytes of certain fishes. Though up to the present time the fish erythrocyte is the most sensitive form of material known, it is not impossible that other types will be discovered in the future of even greater sensitivity. In the meantime, physiologists should constantly be on their guard, when working with sodium chloride, against what, at its worst, is capable of being a source of serious experimental errors.

Summary

1. Of five commercial brands of C.P. sodium chloride that have been studied, one is apparently always harmless and two always destructive to the erythrocytes of certain teleost fishes; one and perhaps both of the others are intermediate and somewhat more variable in their properties.

2. There is indirect evidence that the destructive effect of the toxic brands is due to the presence of an impurity of some sort, which has, however, not been identified. It is not removed by a single recrystallization of the salt.

3. Similar though much less striking differences have been found in the physiological action of the brands of sodium chloride in question upon newly-hatched *Fundulus* and less certainly upon the eggs of *Arbacia*. No constant differences have been noted in the case of mammalian erythrocytes or in that of the cilia of *Mytilus*.

4. It is suggested that in all physiological work in which sodium chloride is used particular attention should be given to the possibility of errors resulting from the presence in the salt of unknown toxic impurities.

Bibliography

Garrey, W. E., 1916. *Am. Jour. Physiol.*, **39**: 313.

Lillie, R. S., 1906. *Am. Jour. Physiol.*, **17**: 89.

Loeb, J., 1900. *Am. Jour. Physiol.*, **3**: 327.

Osterhout, W. J. V., 1922. Injury, Recovery and Death, in Relation to Conductivity and Permeability. Philadelphia.